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Chronoprints: Identifying Samples by Visualizing How They Change over Space and Time

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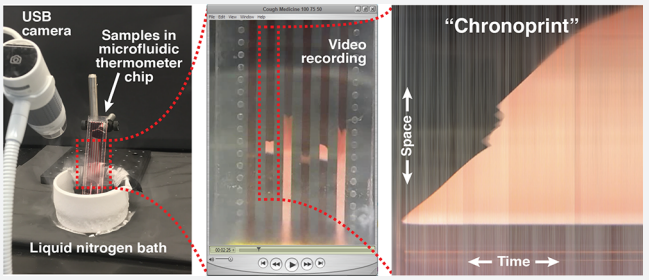
Supporting Information

ABSTRACT: The modern tools of chemistry excel at identifying a sample, but the cost, size, complexity, and power consumption of these instruments often preclude their use in resource-limited settings. In this work, we demonstrate a simple and low-cost method for identifying a sample based on visualizing how the sample changes over space and time in response to a perturbation. Different types of perturbations could be used, and in this proof-of-concept we use a dynamic temperature gradient that rapidly cools different parts of the sample at different rates. We accomplish this by first loading several samples into long parallel channels on a "microfluidic thermometer chip." We then immerse one end of the chip in liquid nitrogen to create a dynamic temperature gradient along the channels, and we use an inexpensive USB microscope to record a video of how the samples respond to the changing temperature gradient. The video is then converted into several bitmap images (one per sample) that capture each sample's response to the perturbation in both space (the *y*-axis; the distance along the dynamic temperature gradient) and time (the *x*-axis); we call these images "chronological fingerprints" or "chronoprints" of each sample. If two samples' chronoprints are similar, this suggests that the samples are the same chemical substance or mixture, but if two samples' chronoprints are significantly different, this proves that the samples are chemically different. Since chronoprints are just bitmap images, they can be compared using a variety of techniques from computer science, and in this work we use three different image comparison algorithms to quantify chronoprint similarity. As a demonstration of the versatility of chronoprints, we use them in three different applications: distinguishing authentic olive oil from adulterated oil (an example of the over $10 billion global problem of food fraud), identifying adulterated or counterfeit medication (which represents around 10% of all medication in low- and middle-income countries), and distinguishing the occasionally confused pharmaceutical ingredients glycerol and diethylene glycol (whose accidental or intentional substitution has led to hundreds of deaths). The simplicity and versatility of chronoprints should make them valuable analytical tools in a variety of different fields.



INTRODUCTION

Techniques for identifying a substance (or the components in a mixture) have many applications across a wide range of different fields. Modern tools of analytical chemistry like gas chromatography−mass spectrometry (GC−MS) are unparalleled in their ability to identify a substance or mixture. However, the size, cost, and complexity of these instruments limit their use in important applications in resource-limited settings. For example, around 10% of all medications in low- and middle-income countries are actually counterfeit and may be worthless (or even dangerous) to patients,[1,2] and while tools like GC−MS could easily detect these adulterated medicines, these tools are not readily available in the poorest parts of the world.

Different substances usually have different physical properties. In some cases, by measuring a physical property of a sample and comparing it to a known value for a pure substance, one can chemically identify the sample. We recently demonstrated two simple and low-cost techniques for measuring two intrinsic physical properties of samples, freezing/melting point[3] and density,[4] and we successfully used those physical measurements to identify some samples. However, many natural products, medicines, and other complex mixtures may not have a known freezing point or density. To identify or distinguish samples like these, simple measurements of their physical properties may not be enough.

In this work, we show that the way a sample's physical properties change over space and time can be used to chemically identify the sample. Under static and homogeneous conditions, a sample's properties usually remain unchanged, so our method relies on inducing a change in the substance by perturbing it in some way. This perturbation could take many different forms, and in this work we used a rapidly changing temperature gradient to perturb our samples. Different samples react to this perturbation in different ways (for example, in a temperature gradient, different samples might freeze, or thaw, or separate into their components, or change in other ways). Additionally, these changes can occur at different *locations* in different samples (if

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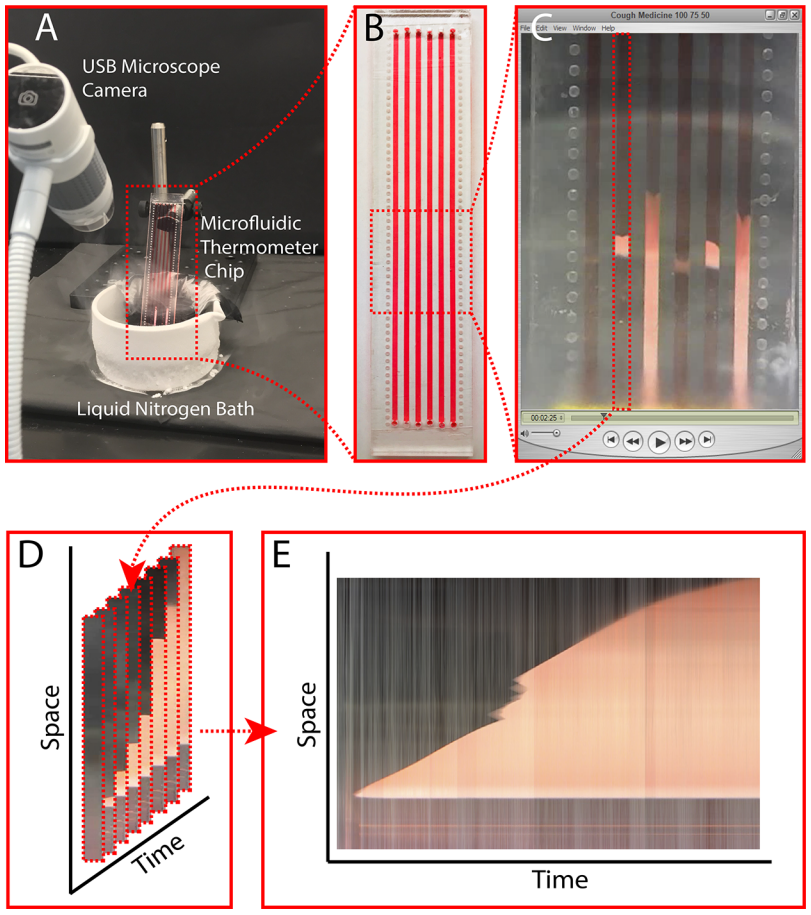

Figure 1. Producing a "chronological fingerprint" or *chronoprint* capturing how six samples (in this example, authentic and adulterated samples of an over-the-counter liquid cold medicine) respond to a perturbation over space and time (in this case, a rapidly changing temperature gradient). (A) A microfluidic thermometer chip containing the samples is partially immersed in liquid nitrogen to establish a rapidly changing temperature gradient along the chip. (B) The chip contains six samples (red) loaded in microfluidic channels that run parallel to the dynamic temperature gradient. (C) An inexpensive USB microscope records a video of the physical changes in the samples as they react to the dynamic temperature gradient. (D) For each sample, our custom MATLAB code (available as Supporting Information) extracts an image of the entire channel from each frame of the video. (E) By reducing each channel image to a single column of pixels, and then placing these columns side-by-side, we create a bitmap image (the sample's chronoprint) that captures how the sample changes over space (the *y*-axis) and time (the *x*-axis). Finally, by comparing the chronoprints of all six samples in the chip, we can determine whether the samples are either likely the same or definitely different.

the perturbation is applied across the sample as a gradient of some sort) and at different *times* in different samples (if the perturbation is changing over time). The resulting multidimensional data set of how a sample changes over space and time in response to a perturbation can serve as a "fingerprint" to identify the sample.

If a perturbation is always applied to a sample in exactly the same manner, then a sample's resulting "fingerprint" should be consistent and could in theory be stored in a database and used to identify the same sample in the future. However, in practice, generating highly reproducible perturbations would likely require complex and costly hardware that would disqualify our technique from use in resource-limited settings. In this work, instead of trying to make the perturbation reproducible across experiments, we made sure that *each sample in a given experiment receives exactly the same perturbation*. In other words, we cannot necessarily compare sample fingerprints across multiple experiments, but we *can* compare fingerprints across multiple samples within the same experiment. We accomplished this using our "microfluidic thermometer chip,"[3] a simple microfluidic chip that holds several different microliter-scale samples in close proximity to each other. The chip (shown in Figure 1A,B) holds several liquid samples in long parallel microfluidic channels. When we apply a perturbation (like a dynamic temperature gradient) along these channels, each sample receives the same perturbation at the same point in space and time. If two samples in the same experiment display similar changes over space and time in response to the perturbation, then this suggests that the samples may be the same. However, if two samples in the same run display significantly different changes over space and time, then this proves that the samples are different.

The data resulting from this process—the change in each sample at each point in space and time as the sample is perturbed—are multidimensional and challenging to analyze, but we developed a simple method for comparing different samples. In this work, we record a movie of the thermometer chip in action, and then convert that movie into bitmap images (one per sample) that capture the way each sample changes over space and time. We call these images "chronoprints," a portmanteau of *chronological* (the image captures a sample's changes over time) and *fingerprint* (the image serves to identify a substance within a given experiment). Figure 1 summarizes the process of obtaining a chronoprint from a complex sample (in this case, six different samples of an over-the-counter cold

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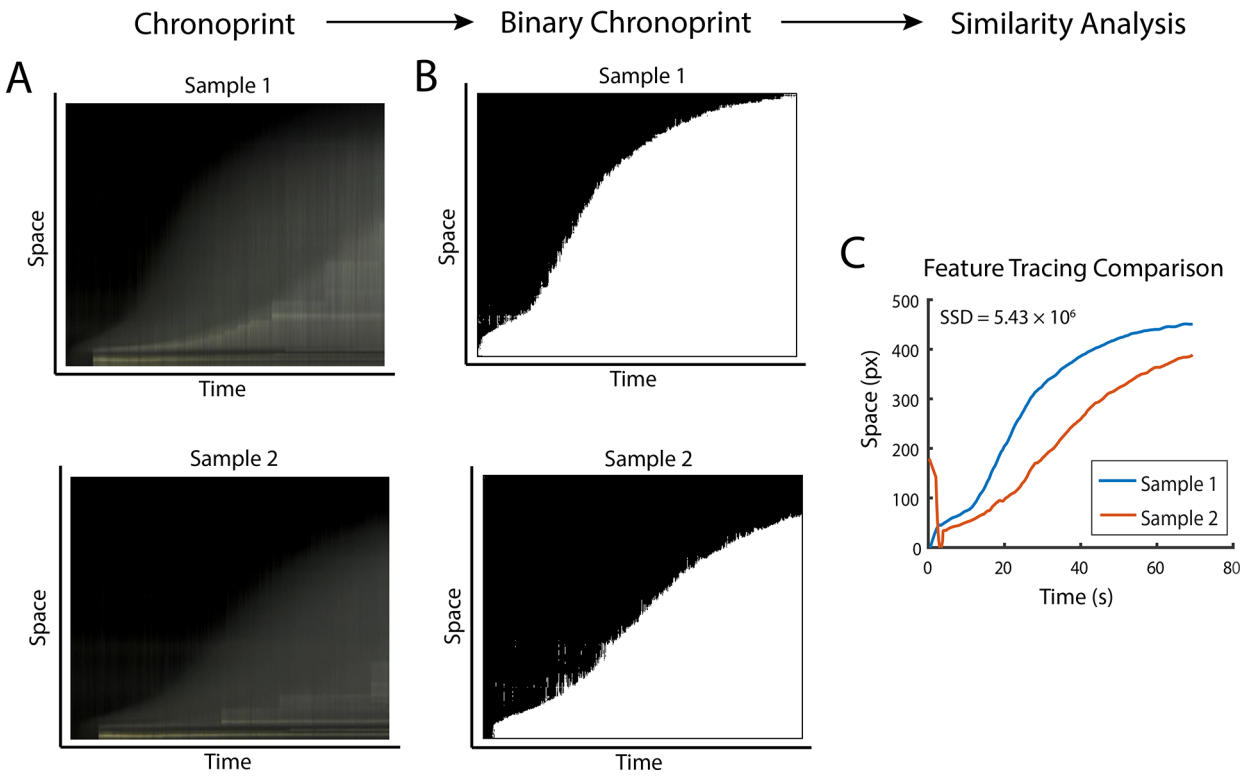

Figure 2. Overview of the *feature tracing* method of comparing chronoprints (in this case, obtained from two different food oils). Monochrome chronoprints of each sample (A) are converted to binary chronoprints (B) by comparing each pixel value to a constant threshold value; pixels above the threshold are colored white, and pixels below the threshold are colored black. Our code (available as online Supporting Information) then traces the boundary between white and black pixels on each binary chronoprint, and the resulting traces are smoothed slightly and plotted together to compare the two chronoprints (C). Traces that are significantly different (like these) confirm that the two samples are chemically different. The sum of squared differences (SSD) between the *y*-axis values of the curves at each point along the curves (5.43×10^6 in this case) serves to quantify the degree of similarity between the two samples of food oils.

medicine exposed to a perturbation consisting of a dynamic temperature gradient). Since a chronoprint is fundamentally just a bitmap image, chronoprints can be compared using image similarity analysis algorithms from computer science. In this work we demonstrate three different approaches to quantifying chronoprint similarity: *feature tracing* (which reduces each chronoprint to a curve and is suitable for simpler chronoprints), *image differences* (which calculates the sum of the pixel-by-pixel differences between two chronoprints), and *image hashing* (which converts each chronoprint to a 64 bit representation called a "hash").

To demonstrate the versatility of chronoprints, we used them here to distinguish between authentic and adulterated foodstuffs, identify adulterated or counterfeit medication, and distinguish between toxic and nontoxic pharmaceutical ingredients. However, these are just a few of the different samples that could be analyzed using chronoprints—in principle any sample that responds to a perturbation by changing its appearance could be analyzed using chronoprints.

EXPERIMENTAL SECTION

Fabricating Microfluidic Thermometer Chips. Microfluidic thermometer chips[3] were designed in Adobe Illustrator (Adobe Systems Inc., San Jose, CA). Each thermometer chip is 125 mm long and 25 mm wide, and contains six parallel microfluidic channels. Each channel is 1.5 mm wide, 0.5 mm deep, and 115 mm long, with 2.5 mm diameter input/output reservoirs at each end, 1.5 mm space between channels, and markers spaced every 1 mm along the sides of the chip for length measurements. The chip design was exported as a DXF file (available as Supporting Information) and engraved into 3 mm thick poly(methyl methacrylate) pieces (Professional Plastics Inc., Fullerton, CA) using a computer-controlled hobbyist-grade milling machine (Bantam Tools, Berkeley, CA). The open channels were enclosed by applying PCR tape (Bio-Rad Laboratories, Hercules, CA) to the chip.

Preparing Samples. Several different types of liquid samples were analyzed in this work. To apply our technique to the problem of counterfeit food products, we obtained chronoprints from samples of two pure food oils, extra virgin olive oil (Wal-Mart Stores Inc., Bentonville, AR) and unrefined peanut oil (Spectrum Organic Products, Petaluma, CA), as well as a 1:1 (v/v) mixture of the two oils that served as an adulterated oil sample. To explore the ability of our technique to determine the authenticity of medications, we obtained chronoprints from six different lots of NyQuil Severe Cold and Flu medicine (a liquid medication containing acetaminophen, phenylephrine, doxylamine succinate, dextromethorphan, and glycerol). The drugs had expiration dates spanning a four month period from July to October 2019. Additionally, to simulate the detection of an adulterated (diluted or watered down) medicine, we prepared and obtained chronoprints from 50%, 75%, 90%, and 95% (v/v) dilutions of NyQuil Severe Cold and Flu medicine in water. Finally, to show that our technique can distinguish two occasionally confused chemicals in pharmaceutical manufacturing, we obtained chronoprints from samples of diethylene glycol (a transparent and sweet-tasting but poisonous liquid) and glycerol (a similar but nonpoisonous

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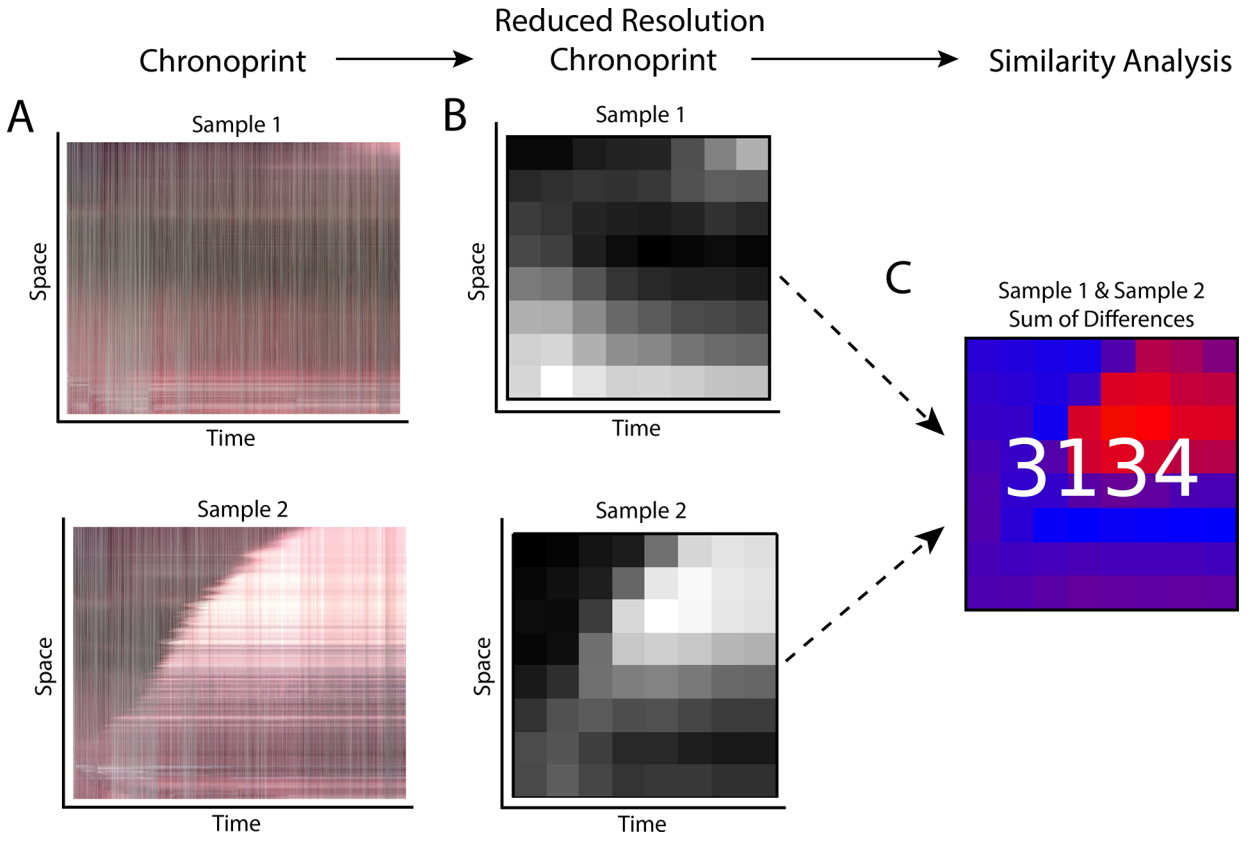

Figure 3. Overview of the *image differences* method of comparing chronoprints (in this case, obtained from authentic and diluted samples of liquid cold medicine). Chronoprints of each sample (A) are converted to reduced-resolution (8 × 8 pixel) monochrome chronoprints (B). The monochrome chronoprints are then compared by calculating the difference between the pixel values at each location; the resulting image (C) shows which regions of the chronoprints are similar (blue) and which are different (red). The sum of these pixel difference values (3134 in this example) quantifies the similarity of these chronoprints on a scale from 0 (completely identical) to 16 320 (completely different). In practice, we found that a threshold of about 1500 generally separates the *image differences* scores of identical substances from different substances, so the *image differences* score of 3134 in this example is significantly greater than 1500 and confirms that these two samples of cold medicine are chemically different.

liquid) from Sigma-Aldrich (St. Louis, MO). About 75 μL of each sample was loaded into the thermometer chip for each experiment.

Obtaining Chronoprints. Once a thermometer chip was filled with samples to analyze (Figure 1B), one end of the chip was partially submerged in a liquid nitrogen bath while recording a video of the chip contents using an inexpensive USB microscope (Figure 1A; Monoprice, Rancho Cucamonga, CA). This created a dynamic temperature gradient that quickly cooled the lower regions of the thermometer chip, and then slowly cooled the rest of the chip over the next few seconds. All six sample channels in the chip were exposed to the same changing temperature gradient. After about 80 s for the oil samples and 160 s for the cold medicine samples, no further changes were observed, and the video recording was ended (Figure 1C). A custom MATLAB script (Supporting Information) was then used to convert each video into six chronoprints (one per sample). For each sample, the script extracts an image of the entire microfluidic channel from each frame of the video (Figure 1D). The script then averages each row of pixels in each channel image to convert it to a single column of pixels. By then placing all of these columns of pixels side-by-side, the script creates a bitmap image that is the sample's chronoprint, with space (distance along the channel) in the vertical dimension and time in the horizontal dimension (Figure 1E). This process is then repeated for each sample in the experiment, and the resulting chronoprints are ready for comparison and similarity analysis.

Comparing Chronoprints. Since chronoprints are just bitmap images, they can be compared using a variety of different techniques, including image similarity algorithms developed by computer scientists.[5] In this work, we used three different techniques to compare chronoprints: *feature tracing*, *image differences*, and *image hashing*. In this Article, we show results from only one comparison technique for each experiment, but results from using the other techniques to analyze these experiments' data (plus several additional experiments' data not shown in the main text) are provided in the Supporting Information.

Comparing Chronoprints Using Feature Tracing. For simpler chronoprints with just one or two dominant features, one can simply trace the boundary between these features and convert each chronoprint to a curve; these curves can then be compared to each other to quantify the similarity of the samples. An example of this *feature tracing* approach for chronoprint comparison is shown in Figure 2. In this process, a custom MATLAB program (Supporting Information) first enhances contrast by taking the pixel values of the first frame (the first column of pixels in the chronoprint), halving these values, and subtracting the result from each remaining column of pixels in the chronoprint. This process helps remove background noise that affects each frame of the movie (and therefore each column

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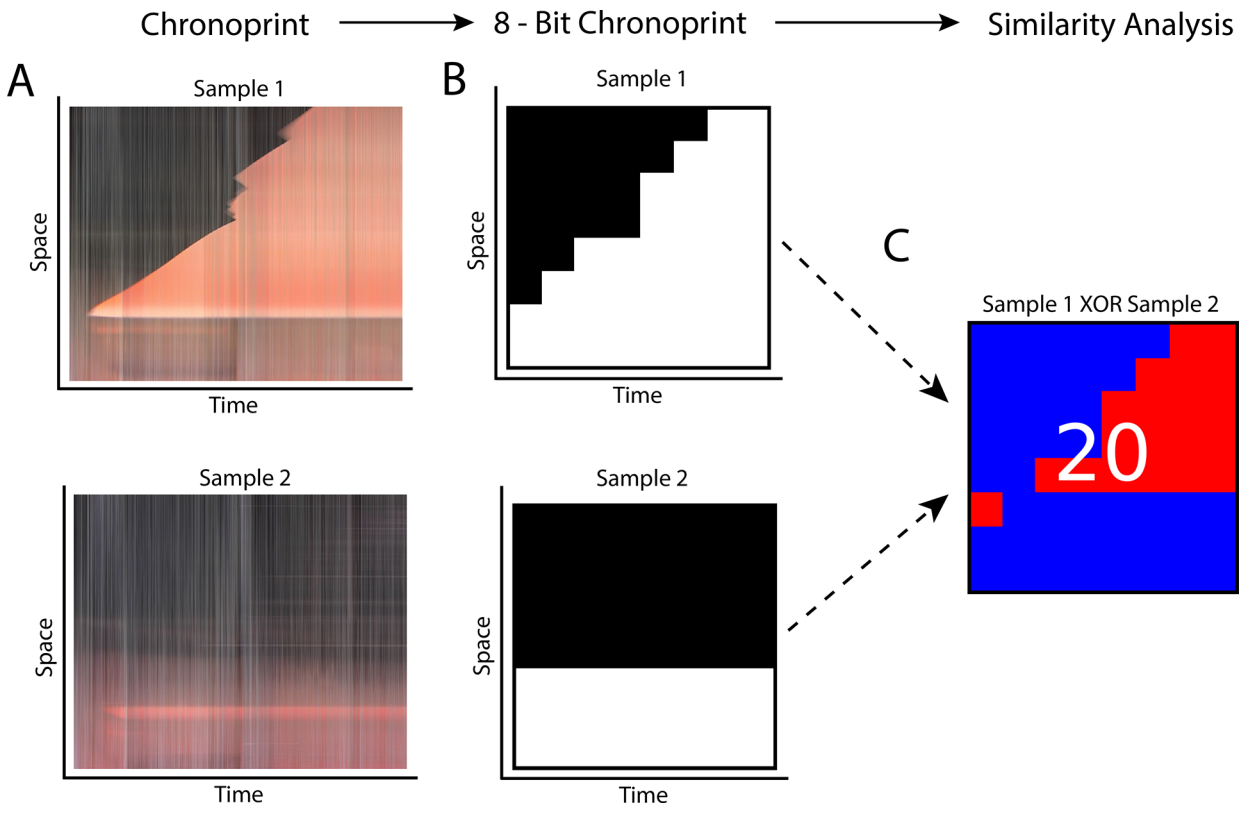

Figure 4. Overview of the *image hashing* method of comparing chronoprints (in this case, obtained from authentic and diluted samples of liquid cold medicine). Chronoprints of each sample (A) are converted to reduced-resolution (8 × 8 pixel) binary chronoprints (B) by comparing each pixel value to a constant threshold. The binary chronoprints are then compared by computing the exclusive OR (XOR) of the pixels at each location in the binary chronoprints, interpreting black = binary "0" or FALSE and white = binary "1" or TRUE. The resulting XOR image (C) is shown with blue pixels wherever the chronoprints are similar and red pixels wherever the chronoprints are different. The number of red pixels in the XOR image, the *image hashing* score (20 in this example), quantifies the degree of similarity of these chronoprints on a scale from 0 (completely identical) to 64 (completely different). In practice, we found that XOR images with more than about 10 red pixels corresponded to chronoprint pairs from different samples, so the *image hashing* similarity score of 20 in this example confirms that these two samples of cold medicine are chemically different.

of the chronoprint) approximately equally. The program then converts each chronoprint from color to monochrome (Figure 2A), and then, the program compares the value of each pixel to a constant threshold provided by the user; pixels with values below that threshold are colored solid black, and pixels with values above that threshold are colored solid white (Figure 2B). The program then traces the boundary between the black and white pixels and converts this trace into a curve (Figure 2C). Rarely, a column of pixels is encountered where the program fails to find the interface between the black and white pixels; in these cases the program reuses the last successful interface location from the previous column of pixels. Finally, the curve is smoothed slightly using a Savitzky-Golay filter[6,7] (third order polynomial; 31 point full window width). If two curves are similar, this suggests that the two samples analyzed may be the same, but if two curves are significantly different, this is proof that the samples are chemically different. The degree of similarity between two samples is quantified by summing the squared differences between the *y*-axis values of the curves (the distances along the channel) at each point along the curves.

Comparing Chronoprints Using Image Differences. While the *feature tracing* method described above works well for simpler chronoprints, more complex chronoprints cannot be easily reduced to simple curves for comparison. For these chronoprints, we compare the bitmap images directly. The *image differences* method for chronoprint comparison calculates the sum of the pixel-by-pixel differences between reduced-resolution versions of two chronoprints. An example of using the *image differences* process is shown in Figure 3. In this process, each chronoprint (Figure 3A) is first converted from color to monochrome, and then, the spatial resolution of each chronoprint is downsampled to 8 by 8 pixels (Figure 3B). Each of the chronoprint's 64 pixels now has a value between 0 (black) and $2^8 - 1 = 255$ (white). To compare two chronoprints, the absolute value of the difference between the pixel values at each pixel location is calculated, and the sum of these values represents the *image difference* score for the two images (Figure 3C). An *image difference* score of 0 indicates that the two chronoprints are exactly identical. The highest possible *image difference* score, $(2^8 - 1) \times 64 = 16\,320$, corresponds to comparing an all-white chronoprint with an all-black chronoprint. In practice, we found that a threshold of about 1500 separated most sample pairs that are identical (*image difference* score <1500) from sample pairs that were different (*image difference* score >1500).

Comparing Chronoprints Using Image Hashing. The third chronoprint comparison method we used, *image hashing*, is shown in Figure 4. This method converts each chronoprint to a reduced-size binary representation (a "hash") that can then be compared to other chronoprints' hashes. The process starts by using the 8 by 8 pixel monochrome version of the chronoprints created in the *image differences* method above. Then, each pixel is converted to either solid white or solid black depending on

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whether its value lies above or below a threshold (Figure 4B). In this study, we explored four different values for this threshold:

- local mean, the average pixel value in each chronoprint was used as the threshold;
- local median, the median pixel value in each chronoprint was used as the threshold;
- global mean, the average pixel value across all six chronoprints in an experiment was used as the threshold;
- global median, the median pixel value across all six chronoprints in an experiment was used as the threshold.

Once a chronoprint is converted to an 8 by 8 binary image, it has effectively been reduced to a 64 bit "hash" of the original chronoprint. To calculate the similarity between two image hashes, our software interprets white pixels as binary "1" or TRUE and black pixels as "0" or FALSE, and then calculates the exclusive OR (also called XOR) of each pixel pair between the images. If two pixels in the same location in two image hashes are the same (that is, they are both white or both black), then the result of the XOR of the pixel values is always 0 (that is, 0 XOR 0 = 0 and 1 XOR 1 = 0). However, if the two pixels are different (if one is black and the other is white), then the result of the XOR of the pixel values is always 1 (that is, 1 XOR 0 = 1 and 0 XOR 1 = 1). By then adding up the sum of all 64 pixel-wise XOR operations, we obtain the two chronoprints' *image hashing* similarity score (Figure 4C). This value ranges from 0 (for two chronoprints with identical image hashes) to 64 (for chronoprints with exactly opposite image hashes).

RESULTS AND DISCUSSION

Identifying Food Fraud. The intentional tampering, substitution, or dilution of food or ingredients, also know as food fraud, is a widespread problem that costs consumers and the food industry from $10 billion to $15 billion a year worldwide.[8] In some cases, food ingredients are substituted or diluted with potentially dangerous or toxic alternates, thereby producing a serious public health concern. For example, in 2008, 22 food companies in China used the toxic compound melamine, commonly used to produce plastic resins, in infant formula to artificially inflate the apparent protein content of their products. This resulted in six infant deaths and nearly 300 000 illnesses.[8−11] In response to the significant economic and health impact of food fraud, the Grocery Manufacturing Association and the United States Congressional Research Service recommend testing food products during and after their production and suggest that authenticating ingredients is the best way to detect adulteration.[8,10]

Olive oil was found to be one of the most commonly adulterated food products worldwide between the years 1980 and 2010,[12] and the University of California, Davis' Olive Center reported in 2010 that 69% of imported olive oils and 10% of California olive oils labeled "extra virgin" did not meet the legal standard.[13] In some cases, "extra virgin" olive oil is diluted with other less expensive oils such as sunflower seed and peanut oils, which pose serious health risks to individuals who are allergic to these foodstuffs.[10,11]

To determine if chronoprints can be used to identify adulterated food oils, we used our technique to analyze various samples of pure oils and oil mixtures. Since these samples resulted in relatively simple chronoprints, we used the *feature tracing* comparison technique to convert each chronoprint into a curve and quantify sample similarity. We began by loading a thermometer chip with six identical samples of 100% extra virgin olive oil before partially submerging the chip in liquid nitrogen, recording a video of the chip as it cools, and converting the video into six chronoprints. The results from performing *feature tracing* analysis on each chronoprint are shown in Figure 5A. Since all six samples were identical, we expected the resulting curves to be very similar, and this is indeed the case. The maximum sum of squared differences between the curves, 4.22×10^5, is relatively low and indicates that the samples are likely identical. In another experiment, we analyzed six identical samples of a different oil

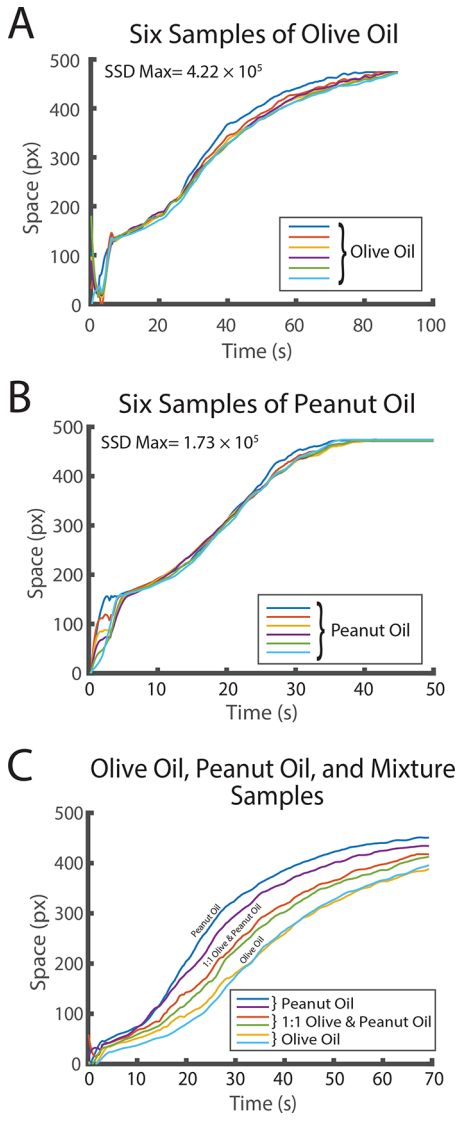

Figure 5. Identifying authentic and adulterated food oils using chronoprints. Each plot compares chronoprints from six food oil samples, converted to curves using the *feature tracing* method. (A) Chronoprint curves from six identical samples of olive oil are nearly identical and differ by a sum of squared differences (SSD) that is 4.22×10^5 or less; this is less than the experimentally observed threshold of 1×10^6 and confirms that the oil samples are identical. (B) Chronoprint curves from six identical samples of peanut oil are similarly identical. (C) Chronoprint curves from two samples each of three different oils (olive oil, peanut oil, and a 1:1 mixture of olive and peanut oil) are similar within each oil type but significantly different between the different oil types. The maximum sum of squared differences between two different oil types (5.77×10^6 difference between the olive oil and peanut oil samples) is greater than the threshold of 1×10^6 and confirms that these oils are different.

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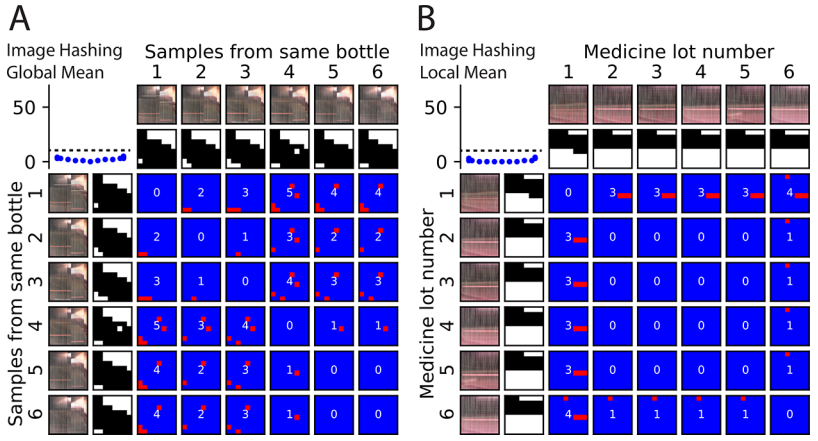

Figure 6. Detecting authentic liquid cold medicine using chronoprints. Each set of images shows six chronoprints along with all pairwise comparisons of the six samples in each experiment, plus a small summary plot of difference scores (blue points = known identical samples). (A) Chronoprints from six identical samples of cold medicine from the same bottle, compared using the *image hashing* method with the global mean pixel value used as the threshold. The resulting image hashes never differ by more than 5 bits; this is well below the 10 bit experimentally observed threshold between identical and different samples (dotted line in summary plot) and confirms that all six medicine samples are identical. (B) Chronoprints from six samples of cold medicine from six different manufacturer's lot numbers, compared using the *image hashing* method with the local mean pixel value used as the threshold. The resulting image hashes never differ by more than 4 bits; this again confirms that the medicine samples are identical (despite having manufacture dates spanning a four month period).

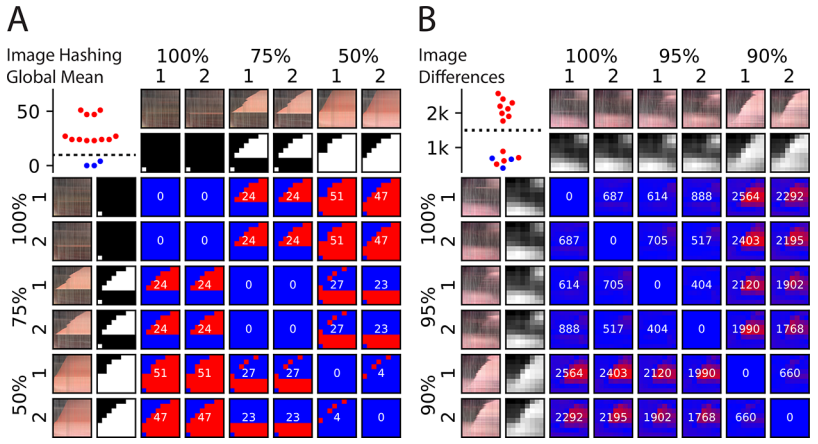

Figure 7. Detecting adulterated liquid cold medicine using chronoprints. (A) Chronoprints from two samples each of three different dilutions of cold medicine in water (50%, 75%, and 100%) again compared using the *image hashing* method. The resulting image hashes successfully confirm that the two samples of each dilution are identical (difference scores of 0, 0, and 4 bits; all <10), and all samples of different dilutions are different (difference scores from 23 to 51 bits; all >10). (B) Chronoprints from two samples each of three additional dilutions of cold medicine (90%, 95%, and 100%) compared using the *image differences* method. The resulting difference images successfully distinguished the 90% samples from the 95% and 100%, with difference scores from 1768 to 2564 (all >1500, the experimentally observed threshold between identical and different samples marked with the dotted line). However, the images failed to distinguish the 100% and 95% samples, with difference scores from 517 to 888 (all <1500 and therefore erroneously identified as identical; red points below the dotted line). Thus, chronoprints are capable of identifying samples of this cold medicine that have been diluted by as little as 10%.

(100% unrefined peanut oil). As expected, the resulting curves are again nearly identical, with a maximum sum of squared difference of 1.73×10^5. These and other results support our claim that all six samples in the microfluidic thermometer receive the same perturbation, and if the samples are identical, then the resulting chronoprints will be very similar (with sum of squared differences between their *feature tracing* curves less than about 1×10^6).

To determine whether different samples produce different chronoprints in the same experiment, we loaded two samples each of three different oils into a thermometer chip. Channels 1 and 4 contained pure peanut oil, channels 3 and 6 pure olive oil, and channels 2 and 5 a 1:1 (v/v) mixture of olive and peanut oils. We then obtained chronoprints for each sample and analyzed them using the *feature tracing* method; the resulting curves are shown in Figure 5C. Within each oil type, each pair of samples resulted in very similar curves: the sum of squared differences was only 5.98×10^4 for the two olive oil samples, 2.55×10^5 for the two peanut oil samples, and 1.04×10^5 for the two olive/peanut mixture samples. However, the different oil types had very different curves: the maximum sum of squared differences was 5.77×10^6 for the olive oil and peanut oil samples. In this and other experiments, we found that chronoprints with *feature tracing* scores greater than about 1×10^6 indicated that the oils were different, and chronoprints with scores less than 1×10^6 indicated that the oils were the same. Additional chronoprints and analysis of experiments with olive oil, peanut oil, and a 1:1

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mixture of the two oil samples are provided in the Supporting Information.

Detecting Counterfeit Medicine. The United Nations estimates that around 10% of all medicines in low- and middle-income countries are counterfeit; consumers waste billions of dollars on these fake drugs every year.[1,2] Simple and inexpensive tools for identifying adulterated drugs can protect consumers from these threats. For example, recent paper-based tests have been developed that can confirm the authenticity of samples of certain drugs.[14−16] However, there remains an unmet need for simple and low-cost techniques that can be applied to a wide range of different types of drugs.

To test the use of chronoprints for distinguishing authentic and adulterated medicine samples, we used our technique to analyze samples of over-the-counter cold medicine. These samples resulted in fairly complex chronoprints, so we used *image differences* and *image hashing* to compare these chronoprints. We first filled a microfluidic thermometer chip with six samples of cold medicine from the same bottle and obtained a chronoprint for each sample. Since these drug samples were identical, we expected that the resulting chronoprints would be very similar. Our experimental results (Figure 6A) confirm this expectation: using *image hashing* with a global mean pixel value as the threshold, all six chronoprints' hashes differ by only 5 or fewer bits out of 64. This small difference in the chronoprints' image hashes confirms that the cold medicine samples are identical. We then filled the chip with six samples of cold medicine from six *different* medicine manufacturer's lots and obtained a chronoprint for each sample. Since these medicine samples are all the same brand, we expected that the resulting chronoprints would also be very similar. Our experimental results (Figure 6B) confirm this expectation: using *image hashing* with a local mean pixel value as the threshold, all six chronoprints' hashes differ by only 4 or fewer bits out of 64. This small difference in the chronoprints' image hashes confirms that these cold medicine samples are also identical, despite being manufactured at different times over a 4 month period. Additional chronoprint experiments, as well as the different analysis methods for the experiments shown in Figure 6, are provided in the Supporting Information.

If a sample of medicine is adulterated by diluting it with water, the sample's chronoprint might change, and this could be the basis of a test to detect adulterated medicines. To test this idea, we filled a microfluidic thermometer chip with two samples each of 50%, 75%, and 100% (v/v) dilutions of cold medicine in water. The resulting chronoprints were again analyzed using *image hashing* with a global mean pixel value as the threshold. The results (Figure 7A) show that, within each dilution, the two samples' chronoprints are identical or nearly so: the two samples of 100% medicine have *identical* image hashes, as do the two samples of 75% medicine, and the two samples of 50% medicine differ by only 4 bits. However, between the different dilutions, the samples' chronoprints were very different: the 100% and 75% dilutions differed by 24 bits, the 75% and 50% dilutions differed by 23 and 27 bits, and the 100% and 50% dilutions differed by 47 and 51 bits. In this and other studies, we found that chronoprint image hashes that differed by more than about 10 bits indicated that the medicines were different (and potentially adulterated), and hashes that differed by less that 10 bits indicated that the medicines were the same. Additional chronoprint experiments for the 50%, 75%, and 100% (v/v) dilutions of cough medicine in water, as well as the different analysis methods for the experiment shown in Figure 7A, are provided in the Supporting Information.

To explore the sensitivity of the chronoprint technique, we repeated the cold medicine analysis in Figure 7A with a smaller difference between the different dilutions: 90%, 95%, and 100% (v/v). For this experiment, we found that the *image differences* comparison method provided the clearest results (Figure 7B). As expected, within each dilution, the two samples' chronoprints are very similar: the two samples of 100% medicine have *image difference* scores of only 687; the two samples of 95% medicine have scores of 404, and the two samples of 90% medicine have scores of 660. These scores are all less than the ∼1500 threshold that we observed separates *image differences* scores of identical (<1500) and different (>1500) samples. Also as expected, two different dilutions' chronoprints were very different: the 90% medicine had *image differences* scores from 2195 to 2564 when compared to the 100% medicine and 1768 to 2120 when compared to the 95% medicine. However, the 95% and 100% medicines had indistinguishable chronoprints—their *image differences* scores ranged from 517 to 888, which are below the ∼1500 threshold and therefore erroneously identified as identical. In summary, the results in Figure 7 show that our chronoprint method can identify samples of this cold medicine that have been diluted by as little as 10%. Additional chronoprint experiments for the 90%, 95%, and 100% (v/v) dilutions of cough medicine in water, as well as the different analysis methods for the experiment shown in Figure 7B, are provided in the Supporting Information.

Identifying Toxic Pharmaceutical Ingredients. In 1937, a chemist at the S. E. Massengill Company in Bristol, Tennessee, unwittingly substituted a toxic substance, diethylene glycol, for nontoxic glycerol in a liquid formulation of the early antibiotic sulfanilamide. The resulting medicine, called "Elixir Sulfanilamide," fatally poisoned over 100 persons,[17,18] and the toxicity of diethylene glycol became common knowledge among pharmaceutical companies. However, remarkably, poisonings due to diethylene glycol in medicines remain tragically common today, with a mass poisoning occurring somewhere in the world on average every two years since 1985.[19] Many of these poisonings occur in resource-limited settings where pharmaceutical companies may not have the resources needed to confirm the identity (and safety) of their manufacturing stocks. The problem of distinguishing diethylene glycol from glycerol is compounded by the fact that they both have very similar properties: they are both transparent, viscous, sweet-tasting liquids, with similar densities, freezing/melting points, and other properties. Consequently, our initial attempts to distinguish diethylene glycol and glycerol by their melting/freezing points alone (using our microfluidic thermometer[3]) were unsuccessful.

To determine whether our chronoprint technique could distinguish toxic diethylene glycol from nontoxic glycerol, we filled a microfluidic thermometer chip with three samples each of both substances, partially immersed the chip in liquid nitrogen, and obtained chronoprints from the video recording of the chip. The chronoprints were then analyzed using the *image hashing* technique with the global mean pixel value used as the threshold. The results, shown in Figure 8, confirm that all the glycerol chronoprint hashes are very similar (never differing by more than 8 bits), as are all the diethylene glycol chronoprint hashes (never differing by more than 10 bits). However, the glycerol chronoprint hashes are significantly different from the diethylene glycol chronoprint hashes (differing by at least 46 bits). These results confirm that our chronoprint method can

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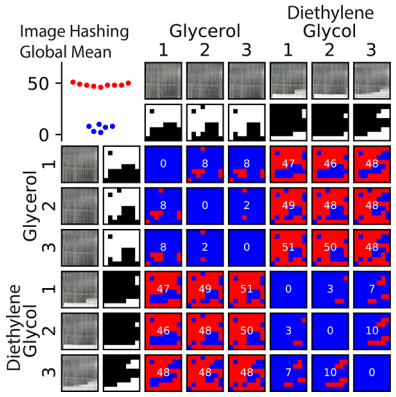

Figure 8. Distinguishing toxic and nontoxic pharmaceutical ingredients using chronoprints. Chronoprints of three samples of toxic diethylene glycol and three samples of nontoxic glycerol were analyzed using the *image hashing* technique with the global mean pixel value as the threshold. The three glycerol chronoprint hashes were nearly identical (differing by 8 or fewer bits), as were the three diethylene glycol hashes (differing by only 3 to 10 bits). However, all of the glycerol chronoprint hashes were significantly different from all of the diethylene glycol hashes (differing by 46 to 51 bits out of a maximum of 64). These results confirm that these substances can be easily distinguished by their chronoprints.

easily distinguish between toxic diethylene glycol and nontoxic glycerol.

CONCLUSION

In this work, we introduced *chronoprints*, an image-based method for identifying or distinguishing substances. Chronoprints are simple and inexpensive to obtain; we used a plastic microfluidic chip, a USB camera, and some liquid nitrogen to obtain ours, but other approaches could be used. In principle, any sample that changes in appearance in response to a perturbation could be analyzed using chronoprints. While we used chronoprints to analyze only liquid samples in this work, the technique is not limited to liquids. For example, solid samples such as pills could be dissolved in constant volumes of water, loaded into the thermometer chip, and analyzed for their authenticity using chronoprints. Even gas mixtures could be analyzed using chronoprints if their components formed condensation or other visible markings on the channel walls of the microfluidic thermometer.

In this proof-of-concept, we used a rapidly changing temperature gradient as the perturbation; this gradient induces phase changes, separations, and other changes within the samples that make for useful chronoprints. However, some samples may be indistinguishable using chronoprints based on dynamic temperature gradients. For example, we could not distinguish the 95% and 100% dilutions of cold medicine using dynamic temperature gradients in Figure 7B. For samples like these, other kinds of perturbations could be used to generate unique chronoprints. For example, if the samples differ in their boiling points, then a high-temperature dynamic temperature gradient might generate different chronoprints for the different samples. For heterogeneous samples containing suspended solids, a changing gravitational acceleration provided by, e.g., a centrifuge may yield unique chronoprints. In general, any perturbation that affects the appearance of different samples in different ways could be the basis for a chronoprint.

Our method does not require that the temperature gradient is reproducible from run to run. Rather, we use multiple parallel microfluidic channels to ensure that each sample experiences the same temperature gradient within a run (thereby enabling comparisons between the samples within that run). This greatly simplifies our technique by eliminating the need for hardware like temperature sensors or controllers. However, if it *were* possible to compare chronoprints from different runs, it would enable us to construct a database of chronoprints for various substances; this would enable our technique to identify a substance without having a known sample of the substance for comparison. One simple way to accomplish this would be to load one or more reference materials into the thermometer chip. For example, materials like temperature-sensitive liquid crystals (whose appearances change in known and reproducible ways as their temperature changes) could serve as internal standards. By scaling a liquid crystal's chronoprint to make it match a reference chronoprint for the liquid crystal, then applying the same scaling to the chronoprints of other samples in the same run, one might obtain "standardized chronoprints" that could be saved to a database and compared between different runs.

One practical limitation to using our chronoprint technique in resource-limited settings is the availability of liquid nitrogen for creating the dynamic temperature gradient. While liquid nitrogen is relatively inexpensive, it requires production and transport infrastructure that may not exist in some locations. For those without access to liquid nitrogen, dry ice may be available from, e.g., carbonated beverage production facilities that are found in many towns. We found that dynamic temperature gradients suitable for obtaining chronoprints can be obtained using dry ice in acetone, although one must first confirm that one's microfluidic thermometer chip material is compatible with the solvent. We also previously used inexpensive Peltier (thermoelectric) coolers to generate temperature gradients,[3] and these coolers could also be used to create chronoprints. Finally, a compressor and freezer coil from an ordinary refrigerator could likely be repurposed to provide the temperature gradient necessary for obtaining chronoprints.

Another practical limitation to our technique concerns the training and other resources required to perform it, but we note that many of the experiments in this work were performed by undergraduate researchers (J.R.-N. and E.D.) who required only minimal training to become proficient at the chronoprint technique. The microfluidic thermometer chip we used required a hobbyist-grade milling machine for fabrication, but we provide the computer design file for this chip in the Supporting Information; we have also demonstrated that consumer-grade 3D printers can be used to fabricate similar chips.[3] Additionally, the MATLAB- and Python-based analysis code we also provide as Supporting Information should simplify the process of generating chronoprints from video recordings of thermometer chips.

Finally, since chronoprints are fundamentally just bitmap images on a computer, we can leverage the enormous variety of image analysis and comparison techniques that have been developed by computer scientists. The image similarity measurements that we used in this proof-of-concept demonstration generally worked well, but they could not algorithmically distinguish the 95% and 100% dilutions of cold medicine in Figure 7B. However, by simply looking at the raw chronoprints in Figure 7B, one can nonetheless see slight differences between the two concentrations' chronoprints. Image comparison algorithms that can recognize these differences will enable our chronoprint technique to correctly identify and discriminate an even wider variety of samples.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00860.

Data from replicate chronoprint experiments, and results from different analysis methods for the experiments shown in the main text (PDF)

Chronoprintgen.m, MATLAB software for converting videos into chronoprints, used to generate all of the chronoprints shown in this work; OilChronoprintAnalysis.m, MATLAB software for analyzing chronoprints using the *feature tracing* method, used to generate Figures 2 and 5 and Figures S1−S3; chronoprint.zip, Python software for analyzing chronoprints using the *image hashing* and *image differences* methods, used to generate Figures 3, 4, and 6−8, and Figures S4−S13; and thermometer_chip.dxf, design of the microfluidic thermometer chip in DXF format, used when milling the chips used in this work on a CNC mill (ZIP)

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Notes

The authors declare no competing financial interest.

Safety statement: liquid nitrogen is a cryogenic hazard and should be handled using appropriate personal protective equipment.

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■ REFERENCES

(1) Blackstone, E. A.; Fuhr, J. P.; Pociask, S. The health and economic effects of counterfeit drugs. *American Health Drug Benefits* **2014**, *7* (4), 216−224.

(2) World Health Organization. *WHO global surveillance and monitoring system for substandard and falsified medical products*; 2017; p 73.

(3) McKenzie, B. A.; Grover, W. H. A microfluidic thermometer: Precise temperature measurements in microliter- and nanoliter-scale volumes. *PLoS One* **2017**, *12*, No. e0189430.

(4) Bhakta, H. C.; Choday, V. K.; Grover, W. H. Musical instruments as sensors. *ACS Omega* **2018**, *3*, 11026−11032.

(5) Gonzalez, R. *Digital image processing*; Prentice Hall: Upper Saddle River, NJ, 2008.

(6) Savitzky, A.; Golay, M. J. Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **1964**, *36*, 1627−1639.

(7) Steinier, J.; Termonia, Y.; Deltour, J. Smoothing and differentiation of data by simplified least square procedure. *Anal. Chem.* **1972**, *44*, 1906−1909.

(8) Grocery Manufacturing Association. *Consumer product fraud: Deterrence and detection*; 2010; p 21.

(9) Everstine, K.; Spink, J.; Kennedy, S. Economically motivated adulteration (EMA) of food: Common characteristics of EMA incidents. *J. Food Prot.* **2013**, *76*, 723−735.

(10) Johnson, R. *Food fraud and economically motivated adulteration of food and food ingredients*; Congressional Research Service, Library of Congress, 2014; p 40.

(11) Hong, E.; Lee, S. Y.; Jeong, J. Y.; Park, J. M.; Kim, B. H.; Kwon, K.; Chun, H. S. Modern analytical methods for the detection of food fraud and adulteration by food category: Adulterated food categories and their analytical methods. *J. Sci. Food Agric.* **2017**, *97*, 3877−3896.

(12) Moore, J. C.; Spink, J.; Lipp, M. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *J. Food Sci.* **2012**, *77*, R118−R126.

(13) Frankel, E.; Mailer, R.; Shoemaker, C.; Wang, S.; Flynn, J. *Tests indicate that imported "extra virgin" olive oil often fails international and USDA standards*; Robert Mondavi Institute for Wine and Food Science; University of California, Davis Olive Center, 2010; p 10.

(14) Weaver, A. A.; Reiser, H.; Barstis, T.; Benvenuti, M.; Ghosh, D.; Hunckler, M.; Joy, B.; Koenig, L.; Raddell, K.; Lieberman, M. Paper analytical devices for fast field screening of beta lactam antibiotics and antituberculosis pharmaceuticals. *Anal. Chem.* **2013**, *85*, 6453−6460.

(15) Koesdjojo, M. T.; Wu, Y.; Boonloed, A.; Dunfield, E. M.; Remcho, V. T. Low-cost, high-speed identification of counterfeit antimalarial drugs on paper. *Talanta* **2014**, *130*, 122−127.

(16) Boehle, K. E.; Carrell, C. S.; Caraway, J.; Henry, C. S. Paper-based enzyme competition assay for detecting falsified beta-lactam antibiotics. *ACS Sensors* **2018**, *3*, 1299−1307.

(17) Geiling, E.; Cannon, P. R. Pathologic effects of elixir of sulfanilamide (diethylene glycol) poisoning: a clinical and experimental correlation. *J. Am. Med. Assoc.* **1938**, *111*, 919−926.

(18) Martin, B. J. *Elixir: The American tragedy of a deadly drug*; Barkerry Press: Lancaster, PA, 2014.

(19) Schep, L. J.; Slaughter, R. J.; Temple, W. A.; Beasley, D. M. G. Diethylene glycol poisoning. *Clin. Toxicol.* **2009**, *47*, 525−35.

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